

Identification of enolases and aldolases as important fish allergens in cod, salmon and tuna: component resolved diagnosis using parvalbumin and the new allergens

A. Kuehn¹, C. Hilger¹, C. Lehnert-Weber², F. Codreanu-Morel², M. Morisset², C. Metz-Favre³, G. Pauli⁴, F. de Blay³, D. Revets⁵, C. P. Muller⁵, L. Vogel⁶, S. Vieths⁶ and F. Hentges^{1,2}

¹Laboratory of Immunogenetics and Allergology, CRP-Santé, Luxembourg, Luxembourg, ²Unit of Immunology and Allergology, Centre Hospitalier de Luxembourg, Luxembourg, Luxembourg, ³Allergy Division, Department of Chest Diseases, University Hospital, Strasbourg, France, ⁴Association pour la Recherche et pour l'Information médicale en Allergologie et en Environnement, Strasbourg, France, ⁵Department of Immunology, CRP-Santé, Luxembourg, Luxembourg and ⁶Department of Allergology, Paul-Ehrlich Institut, Langen, Germany

Clinical & Experimental Allergy

Summary

Background The majority of fish-allergic patients are sensitized to parvalbumin, known to be the cause of important IgE cross-reactivity among fish species. Little is known about the importance of fish allergens other than parvalbumin.

Objective The aim of this study was to characterize hitherto undefined fish allergens in three commonly consumed fish species, cod, salmon and tuna, and to evaluate their importance for *in vitro* IgE-diagnosis in addition to parvalbumin and fish gelatin.

Methods Sixty-two patients were diagnosed by clinical history, skin prick tests and specific IgE to fish extracts. Two new fish allergens from cod, salmon and tuna were identified by microsequencing. These proteins were characterized by immunoblot, ELISA and mediator release assay. Purified parvalbumin, enolase, aldolase and fish gelatin were used for quantification of specific IgE in ELISA.

Results Parvalbumin and two other allergens of 50 and 40 kDa were detected in IgE-immunoblots of cod, salmon and tuna extracts by most patient sera. The 50 and 40 kDa proteins were identified as beta-enolase and fructose-bisphosphate aldolase A respectively. Both purified enzymes showed allergenic activity in the mediator release assay. Indeed, 72.6% of the patients were sensitized to parvalbumin, 20% of these had specific IgE to salmon parvalbumin only. IgE to enolases were found in 62.9% (0.5–95.0 kU_A/L), to aldolases in 50.0% (0.4–26.0 kU_A/L) and to fish gelatin in 19.3% (0.4–20.0 kU_A/L) of the patients. Inter-species cross-reactivity, even though limited, was found for enolases and aldolases by IgE-inhibition ELISA.

Conclusions and Clinical Relevance Fish enolase and aldolase have been identified as important new fish allergens. In fish allergy diagnosis, IgE to enolase and aldolase are especially relevant when IgE to parvalbumin are absent.

Keywords aldolase, allergen, cod, enolase, fish allergy diagnosis, fish gelatin, parvalbumin, salmon, tuna

Submitted 19 July 2012; revised 7 February 2013; accepted 11 February 2013

Correspondence:

Annette Kuehn, Laboratory of Immunogenetics and Allergology, CRP-Santé, 84, Val Fleuri, L-1526 Luxembourg.

E-mail: annette.kuehn@crp-sante.lu

Cite this as: A. Kuehn, C. Hilger, C. Lehnert-Weber, F. Codreanu-Morel, M. Morisset, C. Metz-Favre, G. Pauli, F. de Blay, D. Revets, C. P. Muller, L. Vogel, S. Vieths and F. Hentges, *Clinical & Experimental Allergy*, 2013 (43) 811–822.

Introduction

Fish allergy is one of the most frequent causes for allergic reactions to food throughout the world [1]. The prevalence of fish allergy was estimated in different studies, ranging from < 0.1% to 0.4% in the general population [2, 3]. The incidence of IgE-mediated fish allergy is increased in geographical regions with high fish consumption [4].

Sensitization to fish generally starts during childhood and often, patients remain allergic throughout their life [5]. Clinical manifestations can be mild (oral allergy syndrome, general erythema) to moderate (urticaria, vomiting) or severe (angioedema, asthma, anaphylaxis) [6–8]. Patients may present a single symptom or several clinical manifestations on the same occasion. Symptoms develop upon fish ingestion, but urticaria and eczema

may also occur upon contact with fish and fish products. Fish-allergic patients may have upper and lower airway symptoms through inhalation of odours while preparing or processing fish [9].

Most fish-allergic patients react to multiple fish species, even when they belong to taxonomically distinct fish families [10]. Cross-sensitization of over 80% has been shown by serological studies and skin testing [11, 12]. Clinical cross-reactivity was found to be lower, ranging from 50% to 70% [6, 13, 14]. Differences in allergenicity among fish species were attributed to effects of food processing and variable allergen contents [15–18]. Tuna has been shown to be less allergenic than other fishes [19, 20].

Parvalbumin represents the major fish allergen which has been described in a wide range of fish species [10, 21–23]. In one study, over 90% of the fish-allergic patients were found to have specific IgE to parvalbumins. Parvalbumins are 12 kDa, water-soluble, calcium-binding muscle proteins of remarkable heat stability. Their IgE-binding capacity, thus allergenicity, is reduced upon depletion of calcium-ions [24]. Parvalbumins share a high degree of homology across fish species, which could explain the frequent clinical cross-reactivity in fish-allergic patients [10, 25]. Nevertheless, clinical monosensitivity to parvalbumin has also been reported [26]. On the other hand, cross-reactivity has been shown in clinical cases where the responsible fish allergens were not parvalbumins [27, 28]. Recently, fish gelatin was incriminated in a case of ingestion-induced anaphylaxis [29].

The diagnostic work-up of fish allergy relies on the patient's history, skin prick tests, determination of specific IgE and when indicated, on oral food challenges. A considerable number of fish extracts are available for specific IgE-determination. For several years, recombinant parvalbumins from carp and cod are on hand as *in vitro* diagnostics. A hypoallergenic parvalbumin variant is under investigation for application in immunotherapy for fish allergy [30]. So far, there is no cure available for fish allergy and fish-allergic patients are generally advised to avoid any kind of fish.

Our goal was first to identify and purify hitherto undefined, important fish allergens in three commonly consumed fishes, cod, salmon and tuna. These allergens were then further tested in combination with parvalbumins and fish gelatin for their importance in the diagnosis of fish allergy.

Methods

Fish-allergic patients and sera

Thirty-four patients were recruited from the National Unit of Immunology and Allergology, Luxembourg.

Twenty-eight patients were recruited from inland regions of France in the framework of the allergen task-force of The French Allergology Society (SFA). Criteria for selection were a convincing clinical history of fish allergy, positive skin prick tests (SPT) and specific IgE to cod, salmon and/or tuna extract. The patient population included 37 male and 25 female subjects. Twenty-six children were age 3–12 (mean age 7.6) and 36 adults were age 13–62 (mean age 28.7). Allergic symptoms induced by ingestion, inhalation and contact with fish were recorded. All patients experienced repeatedly clinical symptoms upon exposure to fish. They had positive skin prick tests (SPT) and/or specific IgE higher than 0.35 kU_A/L for cod, salmon or tuna. SPT were performed using commercial extracts (ALK-Abelló, Varannes-en-Argonne, France; Stallergènes, Antony, France) and fresh fish (cod, salmon and tuna). Histamine was used as positive control. A positive SPT response was scored when the wheal diameter was 3–5 mm (+), 6–10 mm (++) or 11–15 mm (+++) larger than the negative control. Serum IgE-antibodies to cod, salmon and tuna extract were measured using the ImmunoCAP system (Phadia-ThermoFisher, Uppsala, Sweden). Levels lower than 0.35 kU_A/L were considered negative. The study was approved by the National Committee for Medical Research Ethics in Luxembourg (Ref. 201001/05) and informed consent was given by all the patients.

Immunoblot analysis with sera from fish-allergic patients

Fish extracts from cod (*Gadus morhua*), salmon (*Salmo salar*) and tuna (*Thunnus albacares*) were prepared as described elsewhere [18]. Immunoblots with extracts were performed as previously described [31]. Briefly, patient sera were diluted 1/5, 1/10 or 1/20 in blocking buffer containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Bornem, Belgium). As negative control, sera from ten non-atopic subjects were used.

Protein sequencing and mass spectrometric analysis

The N-terminal sequences of IgE-reactive 40 and 50 kDa proteins were analysed by automated Edman degradation (Procise 49X HT protein sequencer, Applied Biosystems, Foster City, CA, USA). New protein sequence data of identified 40-kDa aldolases and 50-kDa enolases, respectively, were submitted to the UniProt Knowledgebase for accession numbers.

Identification of enolases and aldolases by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) was performed as described elsewhere [32]. Proteins were identified by peptide mass fingerprints (PMF) after tryptic digestion and by

comparison with the MASCOT 2.0 search engine (Matrix Science, UK) in the NCBI database.

Preparation of fish parvalbumin and gelatin

Parvalbumins from cod, salmon and tuna were purified as previously described [18]. Briefly, fish extracts were separated by ion exchange and size exclusion chromatography. The absence of enolases and aldolases was confirmed by immunoblot using a polyclonal rabbit anti-human enolase (Lifespan Biosciences, Seattle, WA, USA) and a goat anti-human aldolase antibody (Rockland, Gilbertsville, PA, USA). Fish gelatin was prepared using a commercial food-grade product (Rousselot, Courbevoie, France), as described elsewhere [29].

Preparation of fish enolase and aldolase

Enolase and aldolase were purified from cod, salmon and tuna muscle. Total protein was extracted in lysis buffer (50 mM Tris(hydroxymethyl)aminomethane (TRIS)-HCl, pH 8, 150 mM NaCl and 1% Triton X-100) by grinding the muscle tissue in liquid nitrogen. The fish extracts were centrifuged for 10 min at 5300 g to remove insoluble particles. The supernatants were dialysed against 20 mM Tris-HCl, pH 8 (Nanosep 3 kDa; Pall, Zaventem, Belgium). Cod extract proteins were separated by anion exchange chromatography (Resource Q, GE Healthcare, Uppsala, Sweden) using an elution gradient of 0–1 M NaCl in 20 mM Tris-HCl, pH 8. Fractions containing cod enolase or aldolase were dialysed against 20 mM Tris-HCl, pH 8 and applied in two separate runs to a further ion exchange column (Mono Q 5/50 GL; GE Healthcare). Salmon and tuna extract proteins were separated by cation exchange chromatography (Resource S, GE Healthcare) using an elution gradient of 0–1 M NaCl in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6. Fractions containing enolase or aldolase were applied in separate runs to a size exclusion column (Superdex 75 10/300 GL; GE Healthcare) using 50 mM NaH₂PO₄ (pH 7.2) and 150 mM NaCl as running buffer.

Eluted fish enolase and aldolase were concentrated in phosphate-buffered saline (PBS) pH 7.2. Finally, the enolase preparations were supplemented with 0.5 mM MgCl₂. Purities of enolases and aldolases were analysed by analytical sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie G-250 stain (Pierce, Erembodegem, Belgium) or silver stain (SilverSNAP kit, Pierce). Protein identity was confirmed by N-terminal sequencing of the purified enolases and aldolases. In addition, fish enolases or aldolases were identified by immunoblot using the anti-enolase (Lifespan Biosciences) and anti-aldolase antibody (Rockland). The absence of parvalbumin was confirmed using

highly sensitive, polyclonal antibodies raised against cod, salmon and tuna parvalbumin as obtained from a former study [18].

Enzymatic activity and thermal stability

The enolase activity of the purified protein (100–300 µg) was measured by the transformation of 2-phosphoglycerate in a coupled enzymatic assay as described elsewhere [33]. Enzymatic activity of purified aldolases (100–300 µg) was measured by the formation of hydrazone as previously described [34].

Purified enolases and aldolases (500 µg/mL) were incubated at 90°C for 1, 5, 10, 15 and 20 min. Samples were analysed by immunoblot using the anti-enolase (Lifespan Biosciences) and anti-aldolase antibody (Rockland).

Posttranslational modification and oligomeric status

Fish enolases and aldolases were tested for sugar moieties by periodic acid treatment in SDS-PAGE (Glycoprotein staining kit, Pierce). Both purified proteins were analysed for phosphorylation by specific staining in polyacrylamide gel (Phosphoprotein staining kit, Pierce).

The molecular weight of purified enolases and aldolases was determined using a high-performance gel filtration column (Superdex 75 10/300 GL; GE Healthcare) which was calibrated by a gel filtration calibration kit (GE Healthcare).

Mediator release assay

The mediator release assay was performed with a rat basophilic leukaemia (RBL)-2H3 cell line transfected with the α -chain of the human high-affinity IgE receptor, as described elsewhere [35]. Briefly, patient sera were incubated with the cells (clone RBL-703/21) at dilutions 1/10, 1/20 or 1/40 overnight. Antigen-specific release was quantified by measuring the enzymatic activity of β -hexosaminidase. Results were expressed as the percentage of release from cells sensitized with serum in relation to the total release obtained by lysis of the cells. Results were adjusted for spontaneous release (serum added without allergen).

Quantification of specific IgE to parvalbumin, enolase, aldolase and fish gelatin by ELISA

The quantification of specific IgE was performed using enzyme-linked immunosorbent assay (ELISA) as described previously [36]. Details to the ELISA data interpretation are given in Figure S1 in this article's online supporting information. Briefly, purified parvalbumins,

enolases, aldolases and fish gelatin were coated at a concentration of 5 µg/mL. Patient sera were diluted 1/3, 1/5, 1/10, 1/20 or 1/40 in blocking buffer containing 3% BSA. Specific IgE-levels were calculated using the serum of a cat-allergic patient with a known level of specific IgE to cat serum albumin (CSA; Sigma), determined by ImmunoCAP (Phadia). This serum was used in serial dilutions and added to wells coated with CSA. Thus, a standard curve was plotted allowing the calculation of specific IgE-levels to the purified fish allergens. Sera of 25 non-atopic subjects were used as negative controls. The cut-off for positivity was fixed at 0.35 kU_A/L. The mean background from the 25 negative controls was several-fold lower than the cut-off value.

ELISA and ImmunoCAP inhibition experiments

Specific IgE were inhibited by adding the inhibitor allergen to the serum at a final concentration of 200 µg/mL for 2 h at room temperature before using the serum in ELISA. As positive controls, IgE-antibodies were inhibited with each antigen and following, the remaining IgE-reactivity to the same antigen was recorded. To exclude non-significant effects, sera were assayed in the presence of BSA as irrelevant allergen inhibitor. Inhibition of specific IgE to tuna extract (f40) was performed by supplementing patients' sera with tuna parvalbumin at a final concentration of 1 mg/ml and incubating at 4°C overnight.

Statistical methods

Graphpad Prism (version 5.01) was used for data analysis. Data sets were compared using the Mann–Whitney *U*-test for unpaired data and the Fisher's exact test. A value of $P < 0.05$ was considered statistically significant.

Results

Fish-allergic patients

The allergic symptoms presented by the patients upon fish ingestion or contact are detailed in Fig. 1a and Tables S1–S3 in this article's online supporting information. Forty-four patients (71%) reported repeated allergic symptoms upon consumption of different fishes such as cod and salmon and sometimes, tuna, mackerel, trout, sole, sardine, hake or swordfish. Eighteen patients (29%) tolerated single fish species or on the contrary, had allergic symptoms only towards a single fish species. All tested patients were positive in SPT, at least with one of the fishes used in skin testing. Serum IgE-antibodies were positive for cod extract in 79% (49 patients: 0.4–188.0 kU_A/L, median 2.5 kU_A/L) (Fig. 1b),

for salmon extract in 79% (49 patients: 0.4–147.0 kU_A/L, median 2.0 kU_A/L) and for tuna extract in 73% (45 patients: 0.4–77.0 kU_A/L, median 0.9 kU_A/L) of the patients. No correlation was found between the degree of skin reactivity (wheal diameter) and the specific serum IgE-levels.

Selected patient sera with a high IgE-level to tuna extract were inhibited with tuna parvalbumin and then, the remaining IgE-reactivity to tuna extract was quantified. Although IgE-reactivity was considerably reduced, inhibition with parvalbumin did not abolish completely IgE-binding to tuna extract. In patient no. 12, the IgE-level decreased from 25 kU_A/L to 19 kU_A/L and in patient no. 15, from 25 kU_A/L to 15 kU_A/L.

Specific IgE to cod parvalbumin were determined by IgE-ELISA (Tables S1–S3, online supporting information). Remarkably, 26 patients had no specific IgE to cod parvalbumin. These cod parvalbumin-negative patients had symptoms limited to an oral allergy syndrome significantly more often than the patients with specific IgE to cod parvalbumin (Fisher's exact test; $P = 0.0478$). When tested with other parvalbumins, nine of these cod parvalbumin-negative patients were positive with salmon parvalbumin (Table S2, online supporting information).

Identification of enolases and aldolases as fish allergens

When patient sera were tested in immunoblot with cod extract, protein bands were detected in a range from 10 to 60 kDa. Three different pattern of IgE-reactivity were found (Fig. 2a): 15% of the patient sera detected 10-kDa cod parvalbumin and its oligomers (lane 1). Indeed, 54% of the sera detected cod parvalbumin, but also other allergens at 40 and 50 kDa (lane 2). Thirty-one per cent of the sera showed IgE-reactivity to allergens different from cod parvalbumin (lane 3). No IgE-reactive bands were detected using the serum of a birch pollen-allergic patient (lane 4). The 40-kDa and 50-kDa proteins were detected in most IgE-immunoblots. At the same molecular weight, allergen bands were found in salmon and tuna extract (data not shown). N-terminal sequence analysis of the IgE-reactive proteins identified these proteins as fructose-bisphosphate aldolase A and beta-enolase respectively (Table 1).

Biochemical characterization of purified fish enolases and aldolases

Enolases and aldolases from cod, salmon and tuna were purified to homogeneity by chromatography. Protein identity was verified by silver staining. Protein identity was confirmed by immunoblot using a specific anti-enolase and anti-aldolase antibody (Fig. 2b). This was further proven by PMF data analysis with sequence

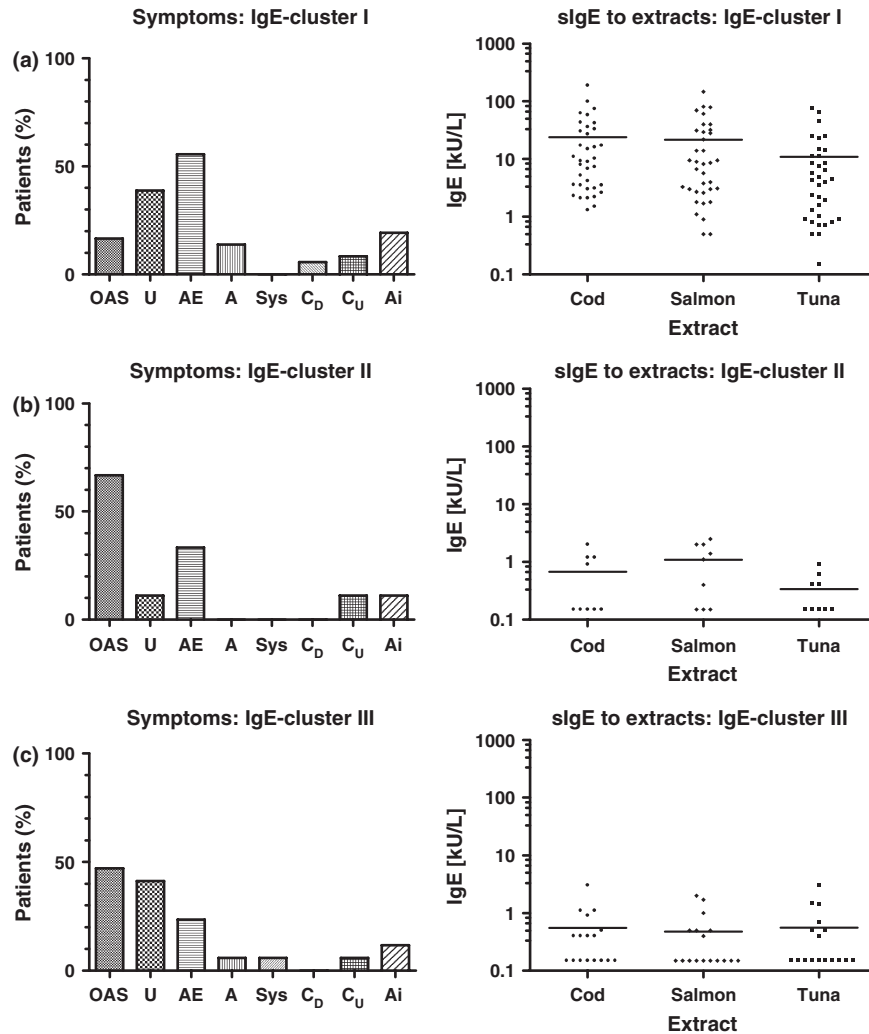


Fig. 1. In the three IgE-clusters, fish-allergic patients showed different characteristics. (a) Cluster I, patients ($n = 36$) had mild to severe symptoms. Mean IgE-levels to fish extracts (Phadia CAP system) were high. (b) Cluster II, patients ($n = 9$) experienced mild symptoms. Most patients had positive, but low IgE-levels to fish extracts. C. Cluster III, patients ($n = 17$) had mild to severe symptoms. Mean IgE-levels to fish extracts were low or mostly, negative. A, asthma (ingestion); Ai, asthma (inhalation); AE, angioedema; C_D, contact dermatitis; C_U, contact urticaria; OAS, oral allergy syndrome; Sys, circulatory collapse; U, urticaria. Horizontal line, mean value.

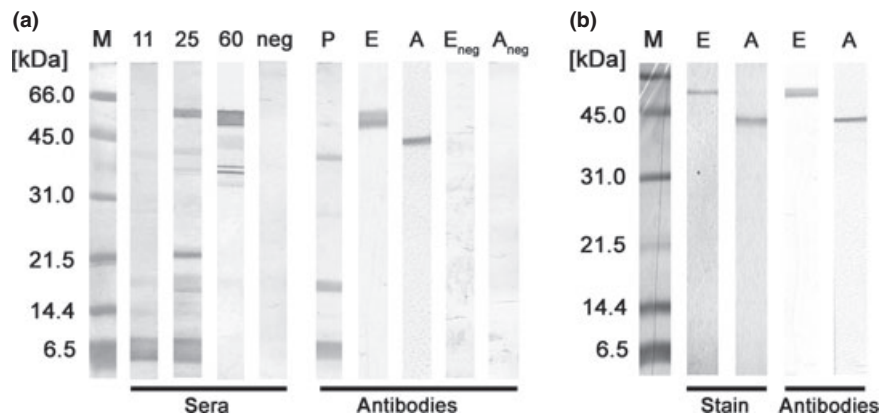


Fig. 2. (a) Cod extract allergens were detected by IgE-binding in patient sera (patient no. 11, 25, 60). Commercial antibodies detected parvalbumin (P), enolase (E) and aldolase (A). M, marker; neg, negative control; E_{neg}, negative control anti-enolase antibody; A_{neg}, negative control anti-aldolase antibody. (b) Purified cod enolase (E) and aldolase (A) were visualized by silver stain and identified by immunoblot.

Table 1. N-terminal sequences of fish enolases and aldolases obtained by microsequencing from IgE-reactive bands in fish extracts and from purified allergens

Fish	Protein band [kDa]	Sequence	Database no.	Protein identity	Allergen
Cod	40	PHAYPFLSPEQKKEL	P86980	Aldolase A	Gad m 3.0101
	50	SITKIKAREIL	B63A0L6	Enolase	Gad m 2.0101
Salmon	5*, 40	AAQEEFIKR, PHAFPFLTPDQKK	B5DGM7	Aldolase A	Sal s 3.0101
	50	SITKIHAREILD	B5DGQ7	Enolase	Sal s 2.0101
Tuna	40	PHAFPFLTPEQKKELSDIAHKIVAPGKGILAADESTG	P86979	Aldolase A	Thu a 3.0101
	30*, 50	GNKLAMQEFMILPVG, SITKIKAREILD	B3A0L7, P86978	Enolase	Thu a 2.0101

*Protein fragments found as minor bands in purified allergen preparations.

coverage of 74%, 85% and 84%, respectively, for cod, salmon and tuna enolase. PMF data analysis matched for cod, salmon and tuna aldolase with sequence coverage of 44%, 48% and 65% respectively. Details of the peptides matching fish enolase and aldolase are shown in Tables S4 to S9 in this article's online supporting information.

Both purified enolase and aldolase were enzymatically active as they converted their respective substrates (data not shown). This confirmed an intact protein folding after chromatographic purification in regards to the catalytic protein site. The IgE-reactivity of purified enolases and aldolases was verified by immunoblot using selected patient sera (data not shown).

Purified enolases turned out to be sensitive to thermal treatment (Fig. S2, online supporting information). Cod and tuna enolase were only detectable in samples heated less than 1 min at 90°C. Salmon enolase was still detected in samples heated for 5 min. Aldolases from cod, salmon and tuna were weakly detected in samples heated for 1–5 min.

No post-translational modifications such as glycosylation and phosphorylation were found for both fish enolase and aldolase (data not shown).

The elution profile of native fish enolases from a calibrated gel filtration column indicated that they are dimers (Fig. S3, online supporting information). In contrast, fish aldolases seemed to be present as oligomeric proteins.

Allergenicity of fish enolases and aldolases

The induction of basophil degranulation by enolases and aldolases was studied using rat basophilic leukaemia cells expressing the human high-affinity IgE receptor and sera from five fish-allergic subjects with high levels of enolase- and aldolase-specific IgE. Both enolases (Fig. 3a) and aldolases (Fig. 3b) induced release with saturation reached at 100 ng/ml. No mediator release was induced when using a pool of sera of three enolase/aldolase-negative patients (no. 11, 43, 56) (Fig. 3) and three pollen-allergic patients (data not shown).

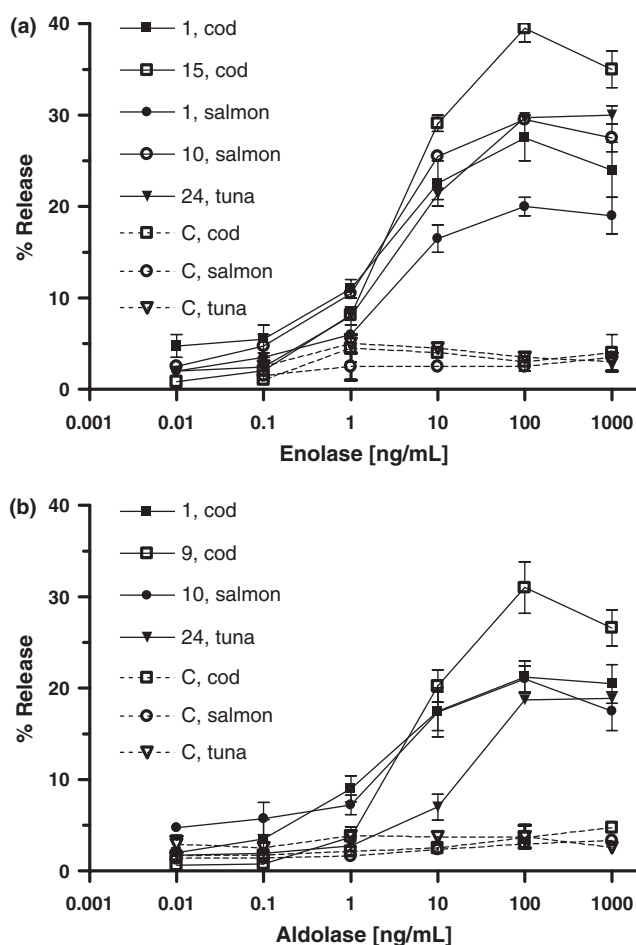


Fig. 3. Mediator release from RBL cells is induced by enolases (a) and aldolases (b) from cod, salmon and tuna. RBL cells were sensitized with sera from five fish-allergic patients (no. 1, 9, 10, 15, 24) and incubated with different concentrations of purified fish enolase or aldolase. C, negative patient serum pool (no. 11, 43, 56). Bars represent means and whiskers indicate standard error of means.

The enolases were designated Gad m 2.0101, Sal s 2.0101 and Thu a 2.0101 by the WHO/IUIS Allergen Nomenclature Sub-Committee (Table 1). The aldolases were named Gad m 3.0101, Sal s 3.0101 and Thu a 3.0101.

Sensitization to fish enolases and aldolases and IgE cross-reactivity

Specific IgE to enolase and aldolase were found in most fish-allergic patients (Tables S1–S3, online supporting information). More precisely, 56% of the patients had serum IgE-antibodies to cod enolase (0.5–95.0 kU_A/L, median 5.0 kU_A/L), 24% had IgE to salmon enolase (0.4–19.0 kU_A/L, median 1.5 kU_A/L) and 19% to tuna enolase (0.6–20.0 kU_A/L, median 2.0 kU_A/L). Indeed, 37% of the subjects had specific IgE to cod aldolase (1.0–22.0 kU_A/L, median 3.0 kU_A/L), 16% had IgE to salmon aldolase (0.4–13.0 kU_A/L, median 4.5 kU_A/L) and 13% to tuna aldolase (0.5–8.0 kU_A/L, median 2.8 kU_A/L).

Seven sera of patients with serum IgE-antibodies to both enolase and aldolase were used in cross-inhibition tests (Fig. 4). In the presence of cod enolase, the IgE-binding for salmon enolase was inhibited by 89–96% in

five of six patients (no. 1, 10, 12, 15, 25). In the same five patients, IgE-binding for tuna enolase was inhibited by 90–96% using cod enolase as inhibitory protein. Cod enolase-specific IgE-antibodies were significantly less inhibited in the presence of salmon enolase (8–33%) and tuna enolase (5–34%), respectively (Mann–Whitney test; $P = 0.0012$ and $P = 0.0070$ respectively), than when using cod enolase as inhibitory protein. IgE-binding of one patient serum (no. 24) was better inhibited by tuna enolase (63–70%) when testing IgE to cod and salmon enolase than by cod enolase (28–31%) when testing IgE to salmon and tuna enolase.

The cross-reactivity among fish aldolases varied greatly among the individual patients. Cod aldolase inhibited 45–85% of IgE-reactivity to salmon aldolase and 9–79% of IgE-reactivity to tuna aldolase. Inhibition of IgE-binding by salmon and tuna aldolase was in a similar range when inhibition was made with fish aldolases. All inhibitions using enolases and aldolases were stronger than inhibitions with BSA (1.1–3.2% and 1.9–3.9% respectively). As positive controls, the self-inhibitions ranged from 94.2 to 97.8% for fish enolases and from 93.5 to 96.9% for aldolases respectively).

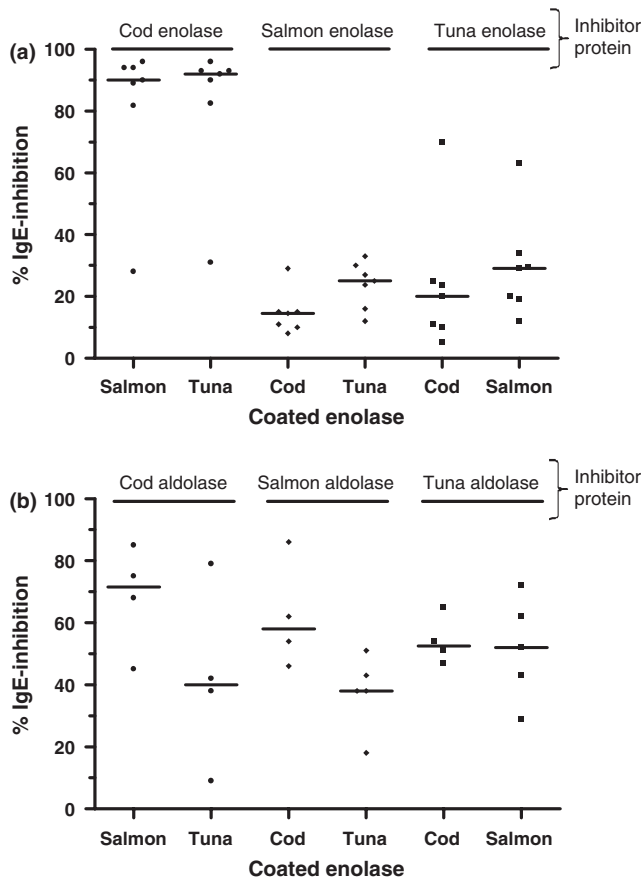


Fig. 4. Serum IgE-antibodies of fish-allergic patients (1, 10, 12, 15, 19, 24, 25) are to some extent cross-reactive to the new fish allergens. (a) IgE-binding to enolases was most efficiently inhibited by cod enolase. (b) IgE to fish aldolase were cross-reactive, but variable among the patients. Cod, cod; S, salmon; T, tuna; horizontal line, mean value.

Diagnostic clusters of fish-allergic patients

The pattern of IgE-binding to parvalbumins was used to divide the fish-allergic patients into three IgE-clusters (Tables S1–S3 in this article's online supporting information and, Figs 1 and 5). In cluster I, patients had IgE to parvalbumin of the three fishes and in most cases also to enolase and aldolase. Patients in cluster II had IgE recognizing parvalbumin from a single fish species, mostly salmon. In cluster III, patients had no specific IgE to parvalbumin, but mostly to enolase, aldolase or fish gelatin. IgE-reactivity to fish gelatin was confirmed by immunoblot using fish skin collagen and fish gelatin (data not shown).

IgE-cluster I

Thirty-six patients (58.1%), 19 children and 17 adults, belonged to this cluster. These patients had specific IgE to cod, salmon and tuna parvalbumin. Twenty-nine patients (80.6%) had IgE-levels to enolases, 21 patients (58.3%) to aldolases and 8 patients (22.2%) to fish gelatin.

All tested patients were positive in SPT with the three target fishes. Four children were not tested in SPT. In cluster I, the patients reacted to multiple fish species such as cod and salmon and sometimes, sole, sardine or hake. Children and adults had mild to severe symptoms. Ingestion-induced atopic dermatitis was diagnosed in 11 patients. Contact allergy was found in five patients and inhalant allergy in seven patients. Four of the patients with respiratory symptoms (no. 7, 12, 19 and

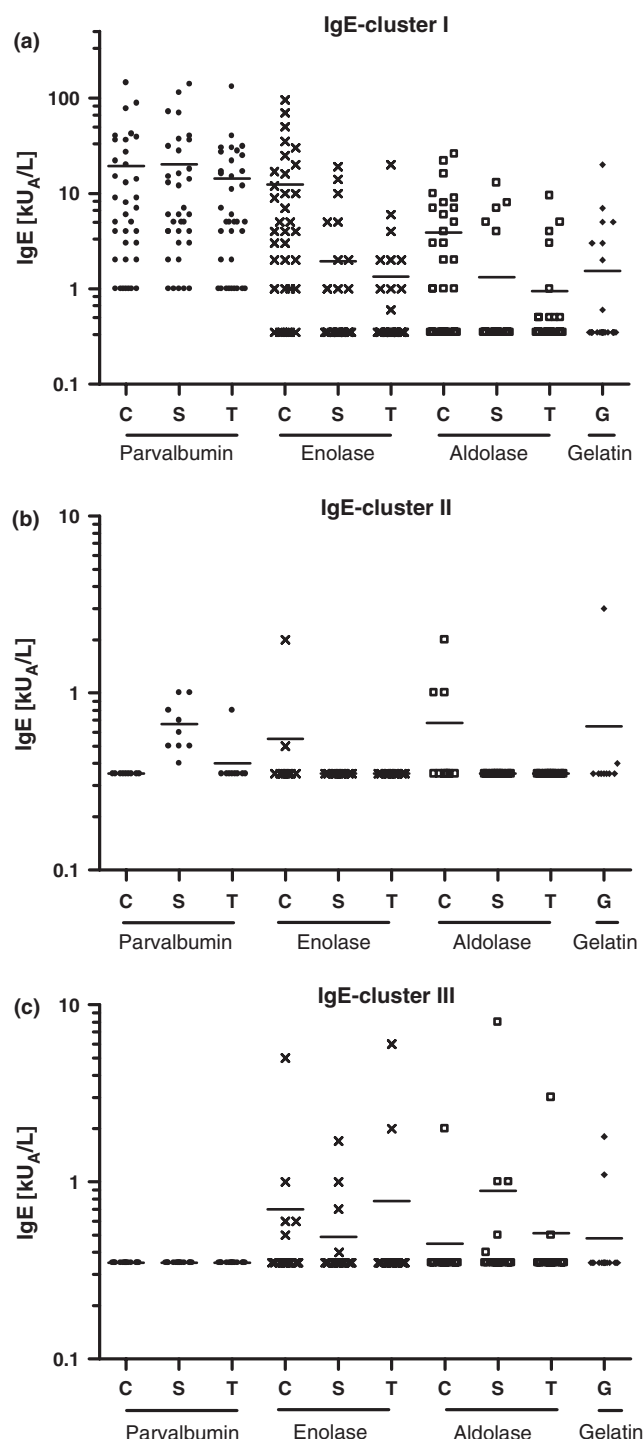


Fig. 5. Fish-allergic patients are grouped in three IgE-clusters. (a) Cluster I, patients have IgE to all parvalbumins and mostly, to other fish allergens. (b) Cluster II, patients have IgE to single parvalbumins, mostly from salmon. (c) Cluster III, patients have no IgE to parvalbumin, but to other allergens. C, cod; G, gelatin; S, salmon; T, tuna; horizontal line, mean value.

21) were sensitized to perennial inhalant allergens, a fact that possibly promoted their asthmatic symptoms upon fish ingestion.

IgE-cluster II

Nine patients (14.5%), two children and seven adults, were grouped to this cluster. These patients had specific IgE to single parvalbumins, eight patients to salmon parvalbumin (0.4–1 kU_A/L, median 0.6 kU_A/L) and one patient to salmon and tuna parvalbumin. Six patients were also positive for other fish allergens (enolase, aldolase, fish gelatin).

All patients were positive in skin tests with at least one fish. In this cluster, five patients indicated to react to single fish species or to tolerate specific fishes. Four patients reacted to other fishes including cod and salmon. Most patients had minor symptoms of fish allergy. Interestingly, patients in IgE-cluster II had significantly more often mild symptoms than patients in cluster I (Fisher's exact test; $P = 0.0275$). A single patient had eczema upon fish ingestion, urticaria upon fish contact and inhalation-induced respiratory problems.

IgE-cluster III

Seventeen patients (27.4%), 5 children and 12 adults, belonged to this IgE cluster. These patients had no specific IgE to parvalbumin from cod, salmon and tuna. Eight patients (47.1%) had specific IgE to fish enolase, and 7 patients (41.2%) had specific IgE to fish aldolase. Fish gelatin was positive in 2 patients (11.8%).

Patients were positive in SPT, but 11 patients had also negative SPT with single fish. Thirteen patients (76%) tolerated single fish species. Children and adults had mild to severe allergic symptoms. One patient had urticaria upon fish contact and two patients had respiratory problems upon inhalation of fish odours.

Discussion

As of today, there is no cure available for fish allergy, fish-allergic patients are commonly advised to avoid consuming all fish. Therefore, an accurate and detailed diagnosis of fish allergy is important. For the diagnosis, the patients' history is confirmed by results from SPT and specific IgE-testing. A panel of fish extracts is available for IgE-based diagnosis. Single recombinant parvalbumins have been reported as a valuable alternative to *in vitro* diagnosis using non-standardized extracts [30]. So far, sensitivity or tolerance of fish-allergic patients to specific fish species can only be confirmed by oral food challenges. Several studies have been carried out in the past, but reliable data are missing about oligo-/monosensitization to single fish species and about the prevalence of sensitization to allergens different from parvalbumin.

In our study, most patients experienced allergic symptoms upon ingestion of several fish species includ-

ing cod and salmon. One third of the patients tolerated single or several fish species. The majority of the patients had specific IgE to fish parvalbumin(s), but 27% were not sensitized to the major fish allergen.

We sought to identify new, important fish allergens in cod, salmon and tuna to improve *in vitro* diagnosis, because these three fishes are among the most commonly consumed in European countries [37]. Cod and salmon regularly trigger important allergic reactions, while tuna is considered to be a hypoallergenic fish species [19, 20]. Absence or low expression levels of tuna parvalbumin might explain this clinical observation [18].

We detected IgE-reactive bands at 10–60 kDa which might be putative fish allergens. We focused on the most frequently recurring protein bands for further analysis. By microsequencing and peptide mass fingerprinting, we identified IgE-reactive proteins of 50 kDa and 40 kDa as beta-enolase and fructose-bisphosphate aldolase A respectively. Recently, enolase has been described as a putative fish allergen in blunt snout bream and tilapia [38, 39]. Aldolase has been identified as IgE-reactive spot by 2D-PAGE in a pacific salmon species [40]. However, sensitization neither to enolase nor to aldolase was confirmed by specific IgE-measurements to these molecules.

We purified enolase and aldolase from cod, salmon, tuna by chromatography. The purified allergens elicited mediator release in rat basophilic leukaemia cells transfected with the α -chain of the human high-affinity IgE receptor, which had been passively sensitized with patient sera. Enolases and aldolases from cod, salmon and tuna as well as fish gelatin, which was previously shown to be responsible for severe anaphylactic reaction [29], were used for IgE-determination in 62 patients. Specific IgE to enolase and aldolase from cod (56.5% and 37.1% respectively) were more frequently found than IgE to the corresponding allergens from salmon and tuna. This could be explained by the higher consumption of cod in our region. On the other hand, the lower sensitization rate to salmon and tuna enolase might result from allergen breakdown by specific preparations such as fish smoking or canning.

IgE cross-reactivity to the new fish allergens was evaluated by cross-inhibition ELISA. Both fish enolases and aldolases showed inter-species cross-reactivity, even though limited. IgE-binding to fish enolases was most efficiently inhibited by cod enolase. This might be explained by a primary sensitization to cod according to eating habits. Concerning the screening of fish allergy, it might be hypothesized whether the detection of enolase-specific IgE is more indicative to verify primary sensitization to a certain fish species than parvalbumin-specific IgE. Cross-reactivity among aldolases was found to be variable in single patients.

Fish enolases and aldolases turned out to be native oligomers which were labile to thermal treatment. Food processing might destroy or create new epitopes. The allergenicity of the new food allergens in authentic food samples would need to be tested in a follow-up study using a larger number of patient sera. Food challenges would allow determining the allergenic potency of the new allergens in patients without PV-specific IgE-antibodies.

The fish-allergic patients under study were divided into three IgE-clusters. In the first IgE-cluster, patients were defined by sensitization to all three parvalbumins. They were allergic to multiple fish such as cod, salmon, sole, hake and sardine. This observation is in line with the generally high IgE cross-reactivity among fish parvalbumins, as reported in the literature [10, 22, 23]. There were more children in cluster I compared to the others clusters, even though not significantly. Interestingly, 80.6% of these patients had also measurable or even high IgE-levels to fish enolase. There was no significant difference between the median IgE-level of cod parvalbumin and cod enolase (Mann-Whitney test; $P = 0.1024$), but between the median IgE-level of salmon and tuna parvalbumin when compared to the corresponding enolases ($P = <0.0001$). 58.3% were positive for fish aldolase. Because of this multiple sensitization, the clinical relevance of the single allergens could not be assessed in these patients.

In the second IgE-cluster, patients had species-specific IgE mostly to salmon parvalbumin (except for patient no. 54 with IgE both to salmon and tuna parvalbumin). The median IgE-level of salmon parvalbumin was significantly lower in cluster II than in cluster I (Mann-Whitney test; $P = <0.0001$). In five of nine patients, the presence of species-specific IgE was correlated with clinical reactivity exclusively to salmon. The symptoms of the patients in this cluster were mild (no asthma, no shock). In this respect, they differ significantly from the patients in cluster I (Fisher's exact test; $P = 0.0275$). Monosensitivity to salmon parvalbumin associated with rather mild symptoms has recently been reported in the literature [26]. It seems to be linked to the presence of IgE directed to an epitope specific for salmon beta 1-parvalbumin [41, 42]. In comparison to cluster I, most of the patients in the second cluster were adults who became sensitized to fish in childhood. Possibly, these patients gained tolerance to single fish species during adolescence.

In the third cluster, 17 patients were allergic to one or several fish species. These patients were not sensitized to parvalbumins. For 12 patients (70.6%), we could detect specific IgE to enolase, aldolase or fish gelatin. The other five patients reacted to still unidentified allergens, which were assumed by the presence of further IgE-reactive bands in immunoblot. Single cases of fish-allergic patients without specific IgE to parvalbumin have been described in the literature [27, 28]. As

27.4% of our patients belong to the third cluster, it might be that these cases are more frequent than previously assumed. Clinical reactions in this cluster varied from mild to severe. Indeed, one patient developed hypotension upon eating fish and another patient developed urticaria, angioedema and asthma after a fish meal, followed by physical effort. The asthmatic symptoms were reproduced after oral challenge with codfish. Both patients were positive for fish aldolase. Thus, fish allergens different from parvalbumin might cause severe reactions. In opposite to IgE-cluster I, most of the patients in cluster III were adults who became allergic to fish in adulthood. It might be analysed in a follow-up study whether allergens different from parvalbumin and especially, enolases and aldolases are more allergenic to adults than to children.

We could show that fish enolases and aldolase are allergens of importance in the population under study, especially in fish-allergic patients not sensitized to parvalbumin. The missing correlation between the degree of skin reactivity and the allergen-specific serum IgE-levels might be explained by low quantities or the absence of enolases and aldolases in commercial extracts. The clinical significance of these allergens should be further assessed in a follow-up study where patients with defined IgE-reactivity profile are food challenged with increasing doses of fish with defined quantities of allergens.

In summary, this study shows that the use of a panel of fish allergens is important for component-resolved diagnosis of sensitization to fish. Even though the use of single recombinant parvalbumin isoforms is sufficient for the diagnosis of highly sensitized patients who

do not tolerate any fish and who have cross-reactive anti-parvalbumin IgE-antibodies, this is not the case for patients who tolerate some fishes. For those patients, the clinical history should be taken into account when choosing the fish extract to be tested *in vitro* or *in vivo*. As cod parvalbumin may be negative in patients sensitized to a single fish species, additional parvalbumins are necessary to complete the panel of fish allergens. A high proportion of our fish-allergic patients were not sensitized to parvalbumin and the use of additional allergens such as enolase, aldolase and possibly fish gelatin is of crucial importance.

Acknowledgements

We thank Dr P Auriol, Dr F Bienvenu, Dr C Braun, Dr C Chepin, Dr A Foessel, Dr L Guenard, Dr P Krieger, Dr JM Renaudin and Dr JF Tuyeras for their collaboration in the framework of the allergen taskforce of the French Allergology Society. We thank T Scheuermann, K Swiontek, P Nazarov and T Graf for technical support.

Funding

This study was supported by the Ministry of Research, Luxembourg.

Conflict of interest

The authors have declared that they have no conflict of interest.

References

- 1 Sicherer SH, Sampson HA. 9. Food Allergy. *J Allergy Clin Immunol* 2006; 117:470–5.
- 2 Rona RJ, Keil T, Summers C *et al.* The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 2007; 120:638–46.
- 3 Taylor SL, Kabourek JL, Hefle SL. Fish allergy: fish and products thereof. *J Food Sci* 2004; 69:175–80.
- 4 Wild LG, Lehrer SB. Fish and shellfish allergy. *Curr Allergy Asthma Rep* 2005; 5:74–9.
- 5 Priftis KN, Mermiri D, Papadopoulou A, Papadopoulos M, Fretzayas A, Lagona E. Asthma symptoms and bronchial reactivity in school children sensitized to food allergens in infancy. *J Asthma* 2008; 45:590–5.
- 6 Helbling A, Haydet R, McCants ML, Musmand JJ, El-Dahr J, Lehrer SB. Fish allergy: is cross-reactivity among fish species relevant? Double-blind placebo-controlled food challenge studies of fish-allergic patients. *Ann Allergy Asthma Immunol* 1999; 83:517–23.
- 7 Sicherer SH, Munoz-Furlong A, Sampson HA. Dose-response in double-blind, placebo-controlled oral food challenges in children with atopic dermatitis. *J Allergy Clin Immunol* 2000; 105:582–6.
- 8 Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to food. *J Allergy Clin Immunol* 2001; 107:191–3.
- 9 Jeebhay MF, Robins TG, Seixas N *et al.* Environmental exposure characterization of fish processing workers. *Ann Occup Hyg* 2005; 49:423–37.
- 10 Van Do T, Elsayed S, Florvaag E, Hordvik I, Endresen C. Allergy to fish parvalbumins: studies on the cross-reactivity of allergens from 9 commonly consumed fish. *J Allergy Clin Immunol* 2005; 116:1314–20.
- 11 Pascual C, Esteban MM, Crespo JF. Fish allergy: evaluation of the importance of cross-reactivity. *J Pediatr* 1992; 121:29–34.
- 12 Bernhisel-Broadbent J, Scanlon SM, Sampson H. Fish hypersensitivity: I. *in vitro* and oral challenge results in fish-allergic patients. *J Allergy Clin Immunol* 1992; 89:730–7.
- 13 De Martino M, Novembre E, Galli L *et al.* Allergy to different fish species in cod-allergic children: *in vivo* and *in vitro* studies. *J Allergy Clin Immunol* 1990; 86:909–14.
- 14 Sicherer SH, Sampson HA. Food allergy. *J Allergy Clin Immunol* 2010; 125:116–25.
- 15 Sletten G, Van Do T, Lindvik H, Egaas E, Florvaag E. Effects of industrial processing on the immunogenicity of commonly ingested fish species. *Int*

- Arch Allergy Immunol* 2010; 15:223–36.
- 16 Lim DL, Neo KH, Goh DL, Shek LP, Lee BW. Missing parvalbumin: implications in diagnostic testing for tuna allergy. *J Allergy Clin Immunol* 2005; 115:874–5.
 - 17 Griesmeier U, Vázquez-Cortés S, Bublin M *et al.* Expression levels of parvalbumins determine allergenicity of fish species. *Allergy* 2010; 65:191–8.
 - 18 Kuehn A, Scheuermann T, Hilger C, Hentges F. Important variations in parvalbumin content in common fish species: a factor possibly contributing to variable allergenicity. *Int Arch Allergy Immunol* 2010; 153:359–66.
 - 19 Bernhisel-Broadbent J, Strause D, Sampson HA. Fish hypersensitivity. II: clinical relevance of altered fish allergenicity caused by various preparation methods. *J Allergy Clin Immunol* 1992; 90:622–9.
 - 20 Pascual CY, Reche M, Fiandor A, Valbuena T, Cuevas T, Martín-Esteban MM. Fish allergy in childhood. *Pediatr Allergy Immunol* 2008; 19:573–9.
 - 21 Lindström CD, van Dô T, Hordvik I, Endresen C, Elsayed S. Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). *Scand J Immunol* 1996; 44:335–44.
 - 22 Beale JE, Jeebhay MF, Lopata AL. Characterisation of purified parvalbumin from five fish species and nucleotide sequencing of this major allergen from Pacific pilchard, *Sardinops sagax*. *Mol Immunol* 2009; 46:2985–93.
 - 23 Perez-Gordo M, Cuesta-Herranz J, Maroto AS *et al.* Identification of sole parvalbumin as a major allergen: study of cross-reactivity between parvalbumins in a Spanish fish-allergic population. *Clin Exp Allergy* 2011; 41:750–8.
 - 24 Bugajska-Schretter A, Elfman L, Fuchs T *et al.* Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion. *J Allergy Clin Immunol* 1998; 101:67–74.
 - 25 Hansen TK, Bindslev-Jensen C, Skov PS, Poulsen LK. Codfish allergy in adults: IgE cross-reactivity among fish species. *Ann Allergy Asthma Immunol* 1997; 78:187–94.
 - 26 Kuehn A, Hutt-Kempf E, Hilger C, Hentges F. Clinical monosensitivity to salmonid fish linked to specific IgE-epitopes on salmon and trout beta-parvalbumins. *Allergy* 2011; 66:299–301.
 - 27 Kondo Y, Komatsubara R, Nakajima Y *et al.* Parvalbumin is not responsible for crossreactivity between tuna and marlin: a case report. *J Allergy Clin Immunol* 2006; 118:1382–3.
 - 28 Ebo DG, Kuehn A, Bridts CH, Hilger C, Hentges F, Stevens WJ. Monosensitivity to pangasius and tilapia caused by allergens other than parvalbumin. *J Investig Allergol Clin Immunol* 2010; 20:84–8.
 - 29 Kuehn A, Hilger C, Hentges F. Anaphylaxis provoked by ingestion of marshmallows containing fish gelatin. *J Allergy Clin Immunol* 2009; 123:708–9.
 - 30 Swoboda I, Bugajska-Schretter A, Linhart B *et al.* A recombinant hypoallergenic parvalbumin mutant for immunotherapy of IgE-mediated fish allergy. *J Immunol* 2007; 178:6290–6.
 - 31 Hilger C, Thill L, Grigioni F *et al.* IgE antibodies of fish allergic patients cross-react with frog parvalbumin. *Allergy* 2004; 59:653–60.
 - 32 Prodhomme EJ, Fack F, Revets D, Pirrotte P, Kremer JR, Muller CP. Extensive phosphorylation flanking the C-terminal functional domains of the measles virus nucleoprotein. *J Proteome Res* 2010; 9:5598–609.
 - 33 Bergmeyer HU. *Methods in enzymatic analysis*. 2nd edn. New York: Academic Press, 1974.
 - 34 Kwon TW, Olcott HS. Tuna muscle aldolase – I. purification and properties. *Comp Biochem Physiol* 1965; 15:7–16.
 - 35 Vogel L, Lüttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005; 60:1021–8.
 - 36 Hilger C, Bessot JC, Hutt N *et al.* IgE-mediated anaphylaxis caused by bites of the pigeon tick *Argas reflexus*: cloning and expression of the major allergen Arg r 1. *J Allergy Clin Immunol* 2005; 115:617–22.
 - 37 Welch AA, Lund E, Amiano P *et al.* Variability of fish consumption within the 10 European countries participating in the European Investigation into Cancer and Nutrition (EPIC) study. *Public Health Nutr* 2002; 5:1273–85.
 - 38 Liu R, Krishnan HB, Xue W, Liu C. Characterization of allergens isolated from the freshwater fish blunt snout bream (*Megalobrama amblycephala*). *J Agric Food Chem* 2011; 59:458–63.
 - 39 Liu R, Yang E, Liu C, Xue W. Tilapia (*Oreochromis mossambicus*) allergens characterized by ELISA, SDS-PAGE, 2D gels, Western blotting and MALDI-TOF mass spectrometry. *Int J Food Sci Nutr* 2011; 63:259–66.
 - 40 Nakamura R, Satoh R, Nakajima Y *et al.* Comparative study of GH-transgenic and non-transgenic amago salmon (*Oncorhynchus masou ishikawae*) allergenicity and proteomic analysis of amago salmon allergens. *Regul Toxicol Pharmacol* 2009; 55:300–8.
 - 41 Perez-Gordo M, Lin J, Bardina L *et al.* Epitope Mapping of Atlantic Salmon Major Allergen by Peptide Microarray Immunoassay. *Int Arch Allergy Immunol* 2011; 157:31–40.
 - 42 Vázquez-Cortés S, Nuñez-Acevedo B, Jimeno-Nogales L, Ledesma A, Fernández-Rivas M. Selective allergy to the Salmonidae fish family: a selective parvalbumin epitope? *Ann Allergy Asthma Immunol* 2012; 108:62–3.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. IgE-levels to fish allergens were determined by an ELISA-based method, as exemplarily

shown for patients no. 7 and 12. Sera dilutions falling into the linear range of the standard curve are shown. A. A standard curve based on CSA-specific IgE-dilutions was used to convert OD-units into $\text{kU}_\text{A}/\text{L}$. Patient sera no. 7 and no. 12 were calculated to have $22 \text{ kU}_\text{A}/\text{L}$

and 27 kU_A/L for cod parvalbumin, respectively. B. OD-values obtained by IgE-reactivity to the different fish allergens are shown. Patient sera no. 7 had specific IgE to fish PV and fish gelatin, but not to the new fish allergens. Patient sera no. 12 had specific IgE to fish PV, fish gelatin, enolases and aldolases (except for cod aldolase). PV, parvalbumin.

Figure S2. Purified allergens were heated for 1, 5, 10 and 15 min and analyzed by immunoblot. Commercial polyclonal antibodies detected enolase (A) and aldolase (B) from cod, salmon and tuna.

Figure S3. Cod enolase eluted as a dimer and cod aldolase as an oligomer from a gel filtration column, as determined by the comparison of the elution volumes (V_e).

Table S1. IgE-cluster I, fish-allergic patients with IgE to parvalbumin from cod, salmon and tuna (children, no. 1–19; adults, no. 20–36).

Table S2. IgE-cluster II, fish-allergic patients with IgE to single parvalbumin (children, no. 37–38; adults, no. 39–45).

Table S3. IgE-cluster III, fish-allergic patients without IgE to fish parvalbumin (children, no. 46–50; adults, no. 51–62).

Table S4. Cod enolase peptides matching with enolase 3 of *Danio rerio* (gi/68086449) sequence after request in the NCBI database.

Table S5. Salmon enolase peptides matching with enolase 3-2 of *Salmo salar* (gi/213513724) sequence after request in the NCBI database.

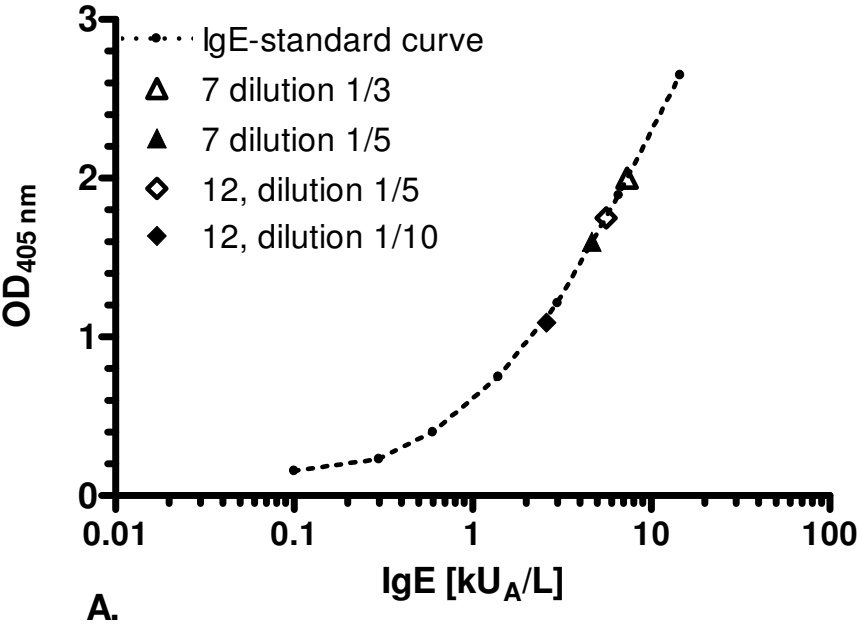
Table S6. Tuna enolase peptides matching with enolase 3-2 of *Salmo salar* (gi/213513724) sequence after request in the NCBI database.

Table S7. Cod aldolase peptides matching with fructose-bisphosphate aldolase A of *Ictalurus punctatus* (gi/308321646) sequence after request in the NCBI database.

Table S8. Salmon aldolase peptides matching with fructose-bisphosphate aldolase A of *Salmo salar* (gi/213511774) sequence after request in the NCBI database.

Table S9. Tuna aldolase peptides matching with fructose-bisphosphate aldolase A of *Salmo salar* (gi/223647884) sequence after request in the NCBI database.

Figure 1-SI.



B.

No.	Allergen	Species	Sera dilution	OD _{405nm}
7	PV	salmon	1/10	1.422
		tuna	1/5	1.313
	fish gelatin	-	1/3	0.908
		cod	1/3	0.002
	enolase	salmon	1/3	0.001
		tuna	1/3	0.008
		cod	1/3	0.003
		salmon	1/3	0.010
		tuna	1/3	0.009
12	PV	salmon	1/10	1.351
		tuna	1/10	1.188
	fish gelatin	-	1/3	1.010
		cod	1/10	1.655
	enolase	salmon	1/3	1.340
		tuna	1/3	0.901
		cod	1/3	0.007
	aldolase	salmon	1/3	1.179
		tuna	1/3	0.859

Figure 2-SI.

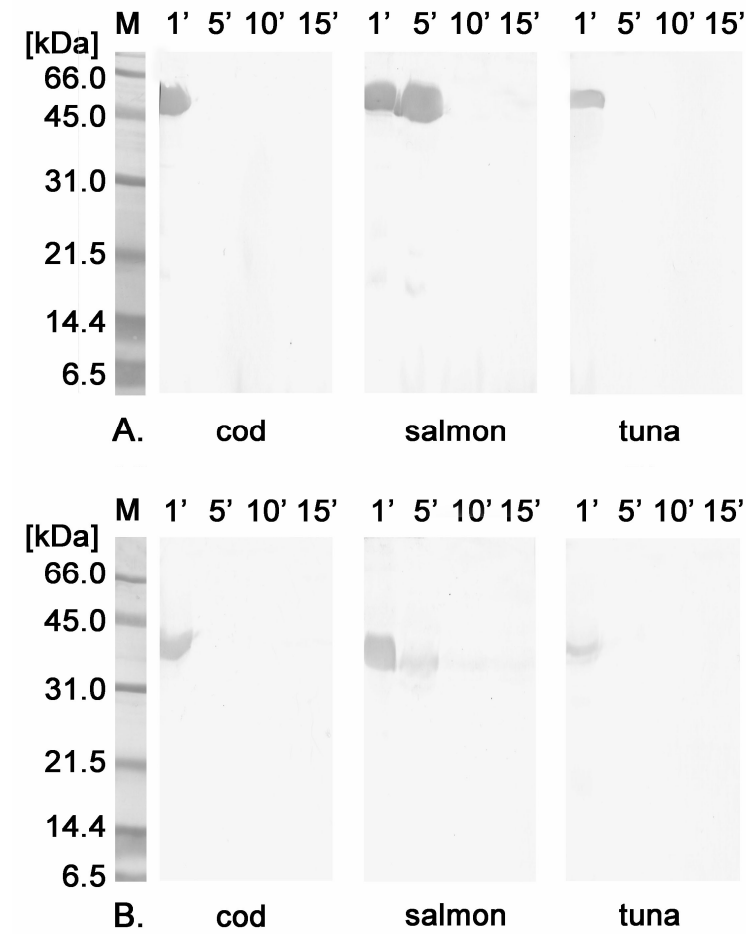
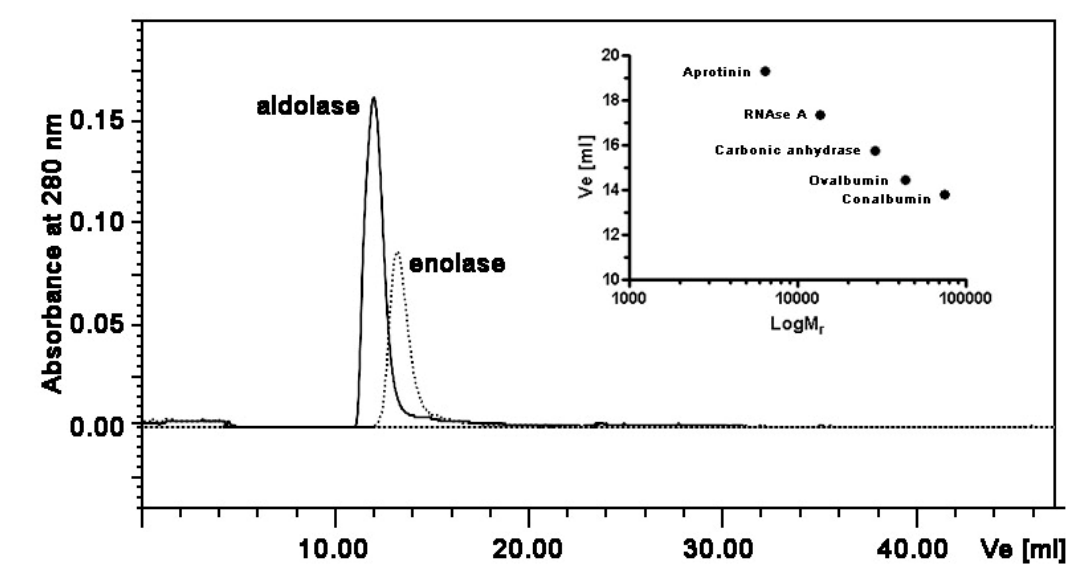


Figure 3-SI.



**Identification of enolases and aldolases as important fish allergens in cod, salmon and tuna:
component resolved diagnosis using parvalbumin and the new allergens.**

Short title: **Importance of fish enolases and aldolases in the diagnosis of fish allergy.**

Annette Kuehn¹, PhD, Christiane Hilger¹, PhD, Christiane Lehnert-Weber², MD, Françoise
Codreanu-Morel², MD, Martine Morisset², MD, PhD, Carine Metz-Favre³, MD, Gabrielle Pauli⁴,
MD, Frédéric de Blay³, MD, Dominique Revets⁵, Claude P Muller⁵, MD, Lothar Vogel⁶, PhD,
Stefan Vieths⁶, PhD and François Hentges, MD^{1,2}

¹Laboratory of Immunogenetics and Allergology, CRP-Santé, Luxembourg, Luxembourg; ²Unit
of Immunology and Allergology, Centre Hospitalier de Luxembourg, Luxembourg,
Luxembourg; ³Allergy Division, Department of Chest Diseases, University Hospital, Strasbourg,
France; ⁴Association pour la Recherche et pour l'Information médicale en Allergologie et en
Environnement, Strasbourg, France; ⁵Department of Immunology, CRP-Santé, Luxembourg,
Luxembourg; ⁶Department of Allergology, Paul-Ehrlich Institut, Langen, Germany

Corresponding author

Annette Kuehn, PhD

Laboratory of Immunogenetics and Allergology

CRP-Santé

84, Val Fleuri

L-1526 Luxembourg

Telephone: + 352 26970 335, Fax: + 352 26970 390

24 E-mail: annette.kuehn@crp-sante.lu

FIGURE LEGENDS

Figure 1-SI. IgE-levels to fish allergens were determined by an ELISA-based method, as exemplarily shown for patients no. 7 and 12. Sera dilutions falling into the linear range of the standard curve are shown. **A.** A standard curve based on CSA-specific IgE-dilutions was used to convert OD-units into $\text{kU}_\text{A}/\text{L}$. Patient sera no. 7 and no. 12 were calculated to have 22 $\text{kU}_\text{A}/\text{L}$ and 27 $\text{kU}_\text{A}/\text{L}$ for cod parvalbumin, respectively. **B.** OD-values obtained by IgE-reactivity to the different fish allergens are shown. Patient sera no. 7 had specific IgE to fish PV and fish gelatin, but not to the new fish allergens. Patient sera no. 12 had specific IgE to fish PV, fish gelatin, enolases and aldolases (except for cod aldolase). PV, parvalbumin.

Figure 2-SI. Purified allergens were heated for 1, 5, 10 and 15 min and analyzed by immunoblot. Commercial polyclonal antibodies detected enolase (**A.**) and aldolase (**B.**) from cod, salmon and tuna.

Figure 3-SI. Cod enolase eluted as a dimer and cod aldolase as an oligomer from a gel filtration column, as determined by the comparison of the elution volumes (V_e).

42 **TABLES**

43 **Table 1-SI.** IgE-cluster I, fish-allergic patients with IgE to parvalbumin from cod, salmon and tuna (children, no. 1-19; adults, no. 20-
 44 36).

Sex/ No. Age Symptoms ¹ SPT ²				IgE by CAP [kU _A /L] ³			IgE by ELISA [kU _A /L] ³									
							parvalbumin			enolase			aldolase			gelatin
				C	S	T	C	S	T	C	S	T	C	S	T	
1	F/3	E, Ai	nd	100	81	44	145	114	131	95	14	4	22	Neg	2.5	Neg
2	M/3	E	Pos	2.3	3.8	1.3	2.5	4.2	4.2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
3	M/4	OAS	Pos	2.1	0.9	Neg	1.2	1.5	0.8	Neg	Neg	Neg	Neg	Neg	Neg	Neg
4	F/5	OAS	Pos	3	3	0.8	3.8	6.5	4.9	1	Neg	Neg	1	Neg	Neg	Neg
5	F/5	OAS	Pos	1.3	1.1	0.5	1.1	0.5	0.4	2.7	Neg	Neg	2	Neg	Neg	Neg
6	M/6	AE, E	Pos	5.2	6.7	4.4	4	5.5	5.4	Neg	Neg	Neg	Neg	Neg	Neg	Neg
7*	F/7	A, AE	Pos	42	40	6.3	22	40.9	16.6	Neg	Neg	Neg	Neg	Neg	Neg	5
8	M/7	U	Pos	2.2	2.6	0.9	2.1	2.9	1.4	1.9	Neg	Neg	Neg	Neg	Neg	Neg
9	F/7	E	Pos	17	14	7.3	1	4	6.1	10	Neg	Neg	26	Neg	Neg	Neg
10	M/7	Ai, AE	nd	188	147	77	88	140	31	70	19	1	Neg	13	1	3
11	F/8	AE	Pos	10.2	9.5	3.9	9	12	11	Neg	Neg	Neg	Neg	Neg	Neg	Neg

12*	M/8	A, AE, E	Pos	36	79	25	27	36	28	50	10	6	Neg	8	4.5	7
13	M/8	AE, U, V	nd	27	22	5.6	36.6	31.6	27.6	12	Neg	Neg	Neg	Neg	Neg	Neg
14	M/8	AE, V	Pos	9	0.5	4.8	15.4	16.8	15.9	20	Neg	Neg	7	Neg	Neg	Neg
15	M/9	AE, U, E	Pos	62	70	25	36	70	30	30	5	1	10	5	4	5
16	F/11	E	Pos	73.9	40	22.5	77	37	25	35	Neg	2	16	Neg	Neg	Neg
17	F/11	OAS	nd	9.2	9.5	4.3	8.4	6.8	5.5	4.1	Neg	Neg	Neg	Neg	Neg	Neg
18	F/11	U	Pos	3.6	3	1.6	1.6	3	2.5	2	Neg	Neg	1	Neg	Neg	Neg
19*	M/12	A, AE, U	Pos	16	32	65	20	18	30	10	5	Neg	3	7	Neg	20
20	F/13	U	Pos	3.5	1.8	1	1	1.6	1.7	3	Neg	2	7	Neg	Neg	1.5
21*	F/13	A, AE	Pos	1.5	1.7	0.7	1.4	1.5	1	1.7	0.7	Neg	1.3	Neg	Neg	Neg
22	F/14	AE, U, C _U	Pos	15	8.6	8.3	9	14	22	Neg	Neg	Neg	Neg	Neg	Neg	Neg
23	F/18	A, AE	Pos	30.2	31.3	14.1	13	15.2	17.2	17	2	Neg	Neg	Neg	Neg	Neg
24	F/21	AE, U	Pos	7.3	5.7	3.5	5	5.1	5.3	7.6	1	20	5	4	9.5	Neg
25	F/21	AE, U	Pos	17	28	8.3	14.3	7.3	4.8	9	1	2	Neg	Neg	Neg	3.1
26	M/21	AE, U	Pos	43	29.4	11	42	28	17	25	Neg	Neg	8	Neg	0.5	Neg
27	M/24	AE, U, Ai	Pos	3.1	3.1	1.9	3.3	3.5	1.8	4.2	1	0.9	Neg	Neg	Neg	0.6
28	M/26	AE, U, Ai, E, C _U	Pos	3.5	0.5	Neg	1.8	0.8	2.7	1	Neg	Neg	1	Neg	Neg	Neg
29	F/27	OAS, Ai	Pos	6.8	4	2.2	5.2	5	5.3	5	Neg	Neg	6	Neg	Neg	Neg
30	M/28	U, E, C _D	Pos	11	9	0.8	6	4	5	4.5	1.5	Neg	9	Neg	Neg	Neg

31	M/31	AE	Pos	7.9	8.2	0.7	7	4.9	1	5	Neg	Neg	2	Neg	Neg	Neg
32	M/31	Ai, E, C _D	Pos	58	61	11	39	72	40	16	Neg	Neg	Neg	Neg	Neg	Neg
33	M/36	OAS	Pos	33	14	15	40	13	12	3.1	Neg	Neg	Neg	Neg	Neg	Neg
34	M/37	AE, E, C _U	Pos	4.2	3.3	2.3	4.1	6.2	7.9	Neg	Neg	Neg	4	Neg	Neg	Neg
35	F/40	AE, U	Pos	2.1	2.7	0.5	2.2	2	1	2	Neg	Neg	2.5	Neg	Neg	Neg
36	M/41	Ai	Pos	2.6	1.8	0.9	2	1.8	1.7	1.5	Neg	0.6	Neg	Neg	Neg	Neg
Mean	-	-	-	23.9	21.7	10.6	19.4	20.5	14.6	12.6	1.9	1.4	3.9	1.3	0.9	1.5
Median	-	-	-	9.1	8.4	4.1	6.5	6.4	5.5	4.2	0.3	0.3	1.0	0.3	0.3	0.3

*, patients sensitized to perennial inhalant allergens (animals, mites, pollen).

¹Symptoms: A, asthma (ingestion); Ai, asthma (inhalation); AE, angioedema; C_D, contact dermatitis; C_U, contact urticaria; E, atopic eczema (contact); OAS, oral allergy syndrome; U, urticaria; V, vomiting.

²SPT: SPT, skin prick test; nd, not done; Pos, positive.

³IgE-determination by Phadia CAP system and ELISA: C, cod; Neg, negative; S, salmon; T, tuna.

51 **Table 2-SI.** IgE-cluster II, fish-allergic patients with IgE to single parvalbumin (children, no. 37-38; adults, no. 39-45).

Sex/ No. Age Symptoms ¹ SPT ²				IgE by CAP [kU _A /L] ³			IgE by ELISA [kU _A /L] ³										
							parvalbumin			enolase			aldolase			gelatin	
				C	S	T	C	S	T	C	S	T	C	S	T		
37*	M/7	OAS	Pos	0.9	2	0.4	Neg	1	Neg	2	Neg	Neg	1	Neg	Neg	Neg	
38*	M/12	U	Pos	Neg	2.5	Neg	Neg	0.8	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	
39	M/14	OAS	Pos	1.2	1.1	0.9	Neg	0.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	
40	M/15	AE	Pos	Neg	1.4	Neg	Neg	0.7	Neg	Neg	Neg	Neg	1	Neg	Neg	Neg	
41*	F/16	OAS	Pos	Neg	Neg	Neg	Neg	0.6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	0.4	
42	M/19	AE	Pos	2	2	0.4	Neg	0.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	3	
43*	F/21	OAS, AE	Pos	Neg	0.4	Neg	Neg	0.4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	
44*	F/26	OAS	Pos	Neg	Neg	Neg	Neg	0.5	Neg	0.5	Neg	Neg	Neg	Neg	Neg	Neg	
45	M/29	Ai, E, OAS, C _U	Pos	1.2	Neg	0.6	Neg	1	0.8	Neg	Neg	Neg	2	Neg	Neg	Neg	
Mean	-	-	-	0.8	1.2	0.5	0.3	0.7	0.4	0.6	0.3	0.3	0.7	0.3	0.3	0.7	
Median	-	-	-	0.3	1.1	0.3	0.3	0.6	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	

52 *, patient tolerates single fish species

53 ¹Symptoms: Ai, asthma (inhalation); AE, angioedema; C_U, contact urticaria; E, atopic eczema (contact); OAS, oral allergy syndrome;
54 U, urticaria.

55 ²SPT: SPT, skin prick test; nd, not done; Pos, positive.

56 ³IgE-determination by Phadia CAP system and ELISA: C, cod; Neg, negative; S, salmon; T, tuna.

57

58 **Table 3-SI.** IgE-cluster III, fish-allergic patients without IgE to fish parvalbumin (children, no. 46-50; adults, no. 51-62).

Sex/ No. Age Symptoms ¹ SPT ²				IgE by CAP [kU _A /L] ³			IgE by ELISA [kU _A /L] ³											
							parvalbumin			enolase			aldolase			gelatin		
				C	S	T	C	S	T	C	S	T	C	S	T			
46*	M/3	U	Pos	0.9	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
47*	M/6	OAS	Pos	1.1	1.7	0.5	Neg	Neg	Neg	Neg	1	Neg	Neg	1	Neg	Neg		
48*	F/8	U	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	0.4	Neg	Neg	Neg	Neg	Neg		
49*	M/10	OAS	Pos	0.4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
50*	M/12	OAS	Pos	0.4	0.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
51	M/14	OAS	Pos	1.1	1	1.4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	8	Neg	Neg		
52*	M/21	A, AE, U	Pos	0.5	0.5	0.7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	0.4	Neg	Neg		
53*	F/22	OAS	Pos	Neg	Neg	Neg	Neg	Neg	Neg	1	Neg	Neg	Neg	Neg	Neg	neg		
54	F/25	OAS, U	Pos	Neg	Neg	Neg	Neg	Neg	Neg	0.6	0.7	Neg	Neg	Neg	Neg	Neg		
55*	M/32	OAS, Ai	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
56	M/34	AE, U	Pos	0.4	0.4	0.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
57*	M/42	Sys	Pos	0.4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	0.5	Neg	Neg		
58*	F/45	Ai, C _U	Pos	Neg	Neg	Neg	Neg	Neg	Neg	0.6	Neg	Neg	Neg	Neg	Neg	Neg		

59*	M/48	AE, U	Pos	Neg	Neg	1.5	Neg	Neg	Neg	Neg	Neg	6	Neg	Neg	0.5	1.8
60	M/54	OAS	Pos	3	2	3	Neg	Neg	Neg	5	Neg	2	2	Neg	3	Neg
61*	M/55	AE	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	1	Neg	Neg
62*	F/60	U	Pos	Neg	0.5	0.4	Neg	Neg	Neg	Neg	1.7	Neg	Neg	Neg	Neg	Neg
Mean	-	-	-	0.6	0.6	0.7	0.3	0.3	0.3	0.7	0.5	0.8	0.4	0.9	0.5	0.5
Median	-	-	-	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3

*, patient tolerates single fish species.

¹Symptoms: Ai, asthma (inhalation); AE, angioedema; C_U, contact urticaria; OAS, oral allergy syndrome; Sys, systemic reaction/circulatory collapse; U, urticaria.

²SPT: SPT, skin prick test; nd, not done; Pos, positive.

³IgE-determination by Phadia CAP system and ELISA: C, cod; Neg, negative; S, salmon; T, tuna.

65 **Table 4-SI.** Cod enolase peptides matching with enolase 3 of *Danio rerio* (gi/68086449) sequence after request in the NCBItr
 66 database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
16-28	1436.746	1435.738	1435.719	0	R.GNPTVEVDLYTTK.G
51-60	1152.619	1151.612	1151.593	2	R.DGDKTRYLGK.G
57-71	1657.844	1656.836	1656.895	2	R.YLGKGTQKAVDHVVK.D
72-81	1154.577	1153.569	1153.707	2	K.DIAPKLIEKK.F
90-103	1652.816	1651.808	1651.813	1	K.IDKFMLELDGTENK.S
121-132	1231.619	1230.612	1230.672	1	K.AGAAEKGVPLYR.H
133-162	3038.676	3037.669	3037.609	1	R.HIADLAGNKDVILPVPAFNVINGGSHAGNK.L
142-162	2119.116	2118.109	2118.122	0	K.DVILPVPAFNVINGGSHAGNK.L
184-193	1143.609	1142.601	1142.608	0	R.IGAEVYHNLK.N
194-202	1020.504	1019.497	1019.613	2	K.NVIKAKYGK.D
200-228	3091.596	3090.589	3090.514	1	K.YGKDATNVGDEGGFAPNILENNEALELLK.S
203-228	2743.379	2742.371	2742.335	0	K.DATNVGDEGGFAPNILENNEALELLK.S
229-253	2700.773	2699.766	2699.388	2	K.SAIEKAGYPDKIIIGMDVAASEFFK.S
254-262	1072.527	1071.520	1071.524	1	K.SGKYDLDFK.S

257-268	1439.756	1438.749	1438.662	1	K.YDLDFKSPDDPK.R
263-281	2129.033	2128.026	2128.080	1	K.SPDDPKRPITGEQLGDLYK.S
286-306	2612.221	2611.213	2611.114	0	K.NYPVQSIEDPFDQDDWENWSK.F
307-334	3061.679	3060.672	3060.555	2	K.FTGSVDIQVVGDDLTVTNPKRIQQACEK.K
336-343	877.518	876.511	876.456	0	K.ACNCLLLK.V
344-358	1576.757	1575.750	1575.793	0	K.VNQIGSVTESIQACK.L
359-372	1541.765	1540.758	1540.757	0	K.LAQSNWGVMSHR.S
395-403	976.471	975.463	975.456	1	K.TGAPCRSER.L
407-412	824.432	823.425	823.401	0	K.YNQLMR.I
407-422	1936.980	1935.973	1935.972	2	K.YNQLMRIIEELGDKAK.F
413-422	1131.589	1130.582	1130.582	1	R.IIEELGDKAK.F

67 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
68 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
69 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

70

71 **Table 5-SI.** Salmon enolase peptides matching with enolase 3-2 of *Salmo salar* (gi/213513724) sequence after request in the NCBI nr
 72 database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
1-9	1056.591	1055.584	1055.591	1	-.MSITKI HAR.E
6-28	2597.350	2596.343	2596.361	2	K.IHAREILDSRGNPTVEVDLYTAK.G
10-28	2120.055	2119.047	2119.080	1	R.EILDSRGNPTVEVDLYTAK.G
10-30	2333.188	2332.181	2332.202	2	R.EILDSRGNPTVEVDLYTAKGR.F
16-32	1922.980	1921.973	1922.001	2	R.GNPTVEVDLYTAKGRFR.A
31-50	2068.065	2067.058	2067.086	1	R.FRAAVPSGASTGVHEALELR.D
33-50	1764.901	1763.894	1763.917	0	R.AAVPSGASTGVHEALELR.D
33-56	2423.211	2422.204	2422.220	2	R.AAVPSGASTGVHEALELRDGDKSR.Y
51-60	1138.591	1137.583	1137.578	2	R.DGDKSRYLGK.G
55-64	1108.633	1107.626	1107.640	2	K.SRYLGKGT VK.A
57-64	865.545	864.537	864.507	1	R.YLGKGT VK.A
65-76	1280.699	1279.692	1279.688	1	K.AVDHVNKDIAAK.L
65-80	1763.798	1762.790	1762.994	2	K.AVDHVNKDIAAKLIEK.K
81-103	2722.338	2721.331	2721.332	2	K.KFSVVDQEKIDHFMLELDGTENK.S

82-89	951.548	950.541	950.471	0	K.FSVVDQEK.I
82-103	2594.259	2593.252	2593.237	1	K.FSVVDQEKIDHFMLELDGTENK.S
82-105	2809.422	2808.415	2808.364	2	K.FSVVDQEKIDHFMLELDGTENKSK.F
104-126	2205.212	2204.205	2204.199	2	K.SKFGANAILGVSLAVCKAGAAEK.G
106-120	1462.854	1461.847	1461.801	0	K.FGANAILGVSLAVCK.A
106-126	1990.119	1989.112	1989.072	1	K.FGANAILGVSLAVCKAGAAEK.G
121-132	1231.664	1230.657	1230.672	1	K.AGAAEKGVPYR.H
133-141	961.602	960.594	960.514	0	R.HIADLAGHK.D
133-162	3065.620	3064.612	3064.566	1	R.HIADLAGHKDVILPCPAFNIVINGGSHAGNK.L
163-183	2406.177	2405.170	2405.170	0	K.LAMQEFMILPIGASNFHEAMR.I
163-193	3530.911	3529.904	3529.767	1	K.LAMQEFMILPIGASNFHEAMRIGAEVYHNLK.N
184-193	1143.621	1142.614	1142.608	0	R.IGAEVYHNLK.N
184-197	1597.919	1596.912	1596.899	1	R.IGAEVYHNLKNVIK.A
200-228	3091.597	3090.590	3090.514	1	K.YGKDATNVGDEGGFAPNILENNEALELLK.T
203-228	2743.376	2742.369	2742.335	0	K.DATNVGDEGGFAPNILENNEALELLK.T
229-253	2730.433	2729.425	2729.399	2	K.TAIEKAGYPDKIIIGMDVAASEFYK.A
234-253	2188.074	2187.067	2187.092	1	K.AGYPDKIIIGMDVAASEFYK.A
240-262	2594.372	2593.364	2593.314	2	K.IIIGMDVAASEFYKAGKYDLDFK.S

254-262	1056.515	1055.507	1055.529	1	K.AGKYDLDFK.S
254-269	1794.845	1793.838	1793.858	2	K.AGKYDLDFKSPDDPAR.Y
263-281	2123.928	2122.921	2123.017	1	K.SPDDPARYITGDQLGDLYK.S
270-281	1385.686	1384.679	1384.687	0	R.YITGDQLGDLYK.S
270-285	1860.941	4859.934	1859.967	1	R.YITGDQLGDLYKSFIK.G
307-326	2103.055	2102.047	2102.089	0	K.FTAAVDIQVVGDDLTVTNPK.R
307-327	2259.164	2258.157	2258.191	1	K.FTAAVDIQVVGDDLTVTNPKR.I
327-334	971.589	970.582	970.556	1	K.RIQQAVEK.K
327-335	1099.647	1098.640	1098.651	2	K.RIQQAVEKK.A
328-334	815.479	814.472	814.455	0	R.IQQAVEK.K
344-355	1274.686	1273.679	1273.688	0	K.VNQIGSVTESIK.A
359-372	1541.760	1540.753	1540.757	0	K.LAQSNWGVVMVSHR.S
404-412	1136.637	1135.630	1135.617	1	R.LAKYNQLMR.I
404-420	2006.049	2005.042	2005.067	2	R.LAKYNQLMRIEEELGAK.A
407-412	824.467	823.460	823.401	0	K.YNQLMR.I
407-420	1693.847	1692.840	1692.850	1	K.YNQLMRIEEELGAK.A
407-422	1892.971	1891.963	1891.983	2	K.YNQLMRIEEELGAKAK.F
421-429	1055.578	1054.570	1054.556	2	K.AKFAGKDYR.H

423-429	856.467	855.460	855.424	1	K.FAGKDYR.H
423-432	1218.645	1217.638	1217.631	2	K.FAGKDYRHPK.I
427-434	1042.559	1041.552	1041.536	2	K.DYRHPKIN.-

73 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
74 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
75 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

76

77 **Table 6-SI.** Tuna enolase peptides matching with enolase 3-2 of *Salmo salar* (gi/213513724) sequence after request in the NCBI nr
 78 database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
1-9	1056.591	1055.584	1055.591	1	-.MSITKI HAR.E
6-28	2597.474	2596.467	2596.361	2	K.IHAREILDSRGNPTVEVDLYTAK.G
10-28	2120.153	2119.146	2119.080	1	R.EILDSRGNPTVEVDLYTAK.G
10-30	2333.289	2332.282	2332.202	2	R.EILDSRGNPTVEVDLYTAKGR.F
16-32	1923.068	1922.061	1922.001	2	R.GNPTVEVDLYTAKGRFR.A
31-50	2068.155	2067.148	2067.086	1	R.FRAAVPSGASTGVHEALELR.D
33-50	1764.983	1763.976	1763.917	0	R.AAVPSGASTGVHEALELR.D
33-56	2423.314	2422.306	2422.220	2	R.AAVPSGASTGVHEALELRDGDKSR.Y
51-60	1138.650	1137.643	1137.578	2	R.DGDKSRYLGK.G
55-64	1108.633	1107.626	1107.640	2	K.SRYLGKGT VK.A
57-64	865.596	864.588	864.507	1	R.YLGKGT VK.A
65-76	1280.760	1279.753	1279.688	1	K.AVDHVNKDIAAK.L
82-89	951.548	950.541	950.471	0	K.FSVVDQEK.I
82-105	2809.552	2808.545	2808.364	2	K.FSVVDQEKIDHFMLELDGTENKSK.F

104-126	2205.275	2204.267	2204.199	2	K.SKFGANAILGVSLAVCKAGAAEK.G
106-120	1462.854	1461.847	1461.801	0	K.FGANAILGVSLAVCK.A
106-126	1990.119	1989.112	1989.072	1	K.FGANAILGVSLAVCKAGAAEK.G
121-132	1231.757	1230.749	1230.672	1	K.AGAAEKGVPPLYR.H
133-141	961.602	960.594	960.514	0	R.HIADLAGHK.D
133-162	3065.764	3064.757	3064.566	1	R.HIADLAGHKDVILPCPAFNIVINGGSHAGNK.L
163-183	2406.281	2405.273	2405.169	0	K.LAMQEFMILPIGASNFHEAMR.I
163-193	3531.029	3530.021	3529.767	1	K.LAMQEFMILPIGASNFHEAMRIGAEVYHNLK.N
184-197	1597.960	1596.953	1596.899	1	R.IGAEVYHNLKNVIK.A
200-228	3091.740	3090.732	3090.514	1	K.YGKDATNVGDEGGFAPNILENNEALELLK.T
203-228	2743.492	2742.485	2742.335	0	K.DATNVGDEGGFAPNILENNEALELLK.T
229-253	2730.545	2729.537	2729.399	2	K.TAIEKAGYPDKIIIIGMDVAASEFYK.A
234-253	2188.172	2187.165	2187.092	1	K.AGYPDKIIIIGMDVAASEFYK.A
240-262	2594.372	2593.364	2593.314	2	K.IIIGMDVAASEFYKAGKYDLDFK.S
254-269	1794.929	1793.921	1793.858	2	K.AGKYDLDFKSPDDPAR.Y
270-281	1385.753	1384.746	1384.687	0	R.YITGDQLGDLYK.S
270-285	1861.033	1860.026	1859.967	1	R.YITGDQLGDLYKSFIK.G
307-327	2259.282	2258.275	2258.191	1	K.FTAAVDIQVVGDDLTVTNPKR.I

327-334	971.627	970.620	970.556	1	K.RIQQAVEK.K
327-335	1099.708	1098.700	1098.651	2	K.RIQQAVEKK.A
328-334	815.527	814.520	814.455	0	R.IQQAVEK.K
344-355	1274.743	1273.736	1273.688	0	K.VNQIGSVTESIK.A
359-372	1541.834	1540.827	1540.757	0	K.LAQSNWGVMSHR.S
404-412	1136.687	1135.679	1135.617	1	R.LAKYNQLMR.I
404-420	2006.140	2005.133	2005.067	2	R.LAKYNQLMRIIEELGAK.A
407-420	1693.932	1692.924	1692.850	1	K.YNQLMRIIEELGAK.A
407-422	1893.055	1892.047	1891.983	2	K.YNQLMRIIEELGAKAK.F
421-429	1055.625	1054.618	1054.556	2	K.AKFAGKDYR.H
423-429	856.518	855.511	855.424	1	K.FAGKDYR.H
423-432	1218.706	1217.699	1217.631	2	K.FAGKDYRHPK.I
427-434	1042.614	1041.607	1041.536	2	K.DYRHPKIN.-

79 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
80 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
81 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

82

83 **Table 7-SI.** Cod aldolase peptides matching with fructose-bisphosphate aldolase A of *Ictalurus punctatus* (gi/308321646) sequence
 84 after request in the NCBI database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
1-13	1544.7363	1543.7290	1543.7493	0	-.MPHAYPFLSPEQK.K
2-13	1413.6159	1412.6087	1412.7088	0	M.PHAYPFLSPEQK.K
2-14	1541.6740	1540.6668	1540.8038	1	M.PHAYPFLSPEQKK.E
15-22	916.4285	915.4212	915.5025	0	K.ELSDIALR.I
29-42	1318.6143	1317.6070	1317.6776	0	K.GILAADESTGSVAK.R
61-87	3138.3799	3137.3727	3137.6371	1	R.QLLFTADDRVKPCIGGVIFFHETLYQK.T
109-134	2690.2312	2689.2240	2689.3558	1	K.VDKGVVPLAGTNGETTTQGLDGLYER.C
112-134	2348.0390	2347.0318	2347.1655	0	K.GVVPLAGTNGETTTQGLDGLYER.C
141-149	1065.4185	1064.4112	1064.5039	1	K.DGADFAKWR.C

85 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
 86 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
 87 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

88

89 **Table 8-SI.** Salmon aldolase peptides matching with fructose-bisphosphate aldolase A of *Salmo salar* (gi/213511774) sequence after
 90 request in the NCBI nr database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
2-13	1397.8113	1396.8040	1396.7139	0	M.PHAFPFLTPDQK.K
2-14	1525.9056	1524.8984	1524.8089	1	M.PHAFPFLTPDQKK.E
15-22	888.6248	887.6176	887.4964	0	K.ELSDIALK.I
27-42	1503.9443	1502.9370	1502.7940	1	K.GKGILAADESTGSVAK.R
29-43	1474.9075	1473.9003	1473.7787	1	K.GILAADESTGSVAKR.F
44-56	1581.8448	1580.8375	1580.7066	0	R.FQSINTENTEENR.R
44-57	1737.9131	1736.9059	1736.8077	1	R.FQSINTENTEENRR.L
61-69	1078.6528	1077.6455	1077.5455	0	R.QLLFTADDR.A
88-99	1329.7737	1328.7664	1328.6724	1	K.TDAGKTFPEHVK.S
93-99	857.5449	856.5376	856.4443	0	K.TFPEHVK.S
100-108	1001.7015	1000.6943	1000.5817	1	K.SRGWVVGK.V
102-111	1100.7367	1099.7294	1099.6389	1	R.GWVVGKVDK.G
109-134	2690.3797	2689.3725	2689.3558	1	K.VDKGVVPLAGTNGETTTQGLDGLYER.C
112-134	2348.2051	2347.1978	2347.1655	0	K.GVVPLAGTNGETTTQGLDGLYER.C

141-149	1097.6085	1096.6013	1096.4760	1	K.DGCDFAKWR.C
202-215	1644.9708	1643.9635	1643.8592	1	R.TQYVTEKVLAAAMYK.A
244-258	1704.9834	1703.9761	1703.8665	0	K.YTHQEIAMATVTALR.R
244-259	1861.0600	1860.0527	1859.9676	1	K.YTHQEIAMATVTALRR.T
313-322	971.5989	970.5916	970.4984	0	K.AWGGKPGNGK.A
323-330	935.6102	934.6029	934.4760	0	K.AAQEEFIK.R
323-331	1091.6879	1090.6807	1090.5771	1	K.AAQEEFIK.R.A
331-342	1231.7572	1230.7500	1230.6502	1	K.RALANSLACQGK.Y
332-342	1075.6586	1074.6513	1074.5491	0	R.ALANSLACQGK.Y

91 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
92 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
93 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

94

95 **Table 9-SI.** Tuna aldolase peptides matching with fructose-bisphosphate aldolase A of *Salmo salar* (gi/223647884) sequence after
 96 request in the NCBI database.

Start-End	Observed	Mr, exp.	Mr, calc.	Miss	Sequence
1-14	1676.903	1675.896	1675.839	1	-.MPHSFPFLSTEQKK.E
2-13	1417.724	1416.716	1416.704	0	M.PHSFPFLSTEQK.K
23-42	1884.028	1883.0205	1883.036	1	K.IVAPGKGILAADESTGSVAK.R
23-43	2040.149	2039.142	2039.137	2	K.IVAPGKGILAADESTGSVAKR.F
43-57	1863.914	1862.907	1862.898	2	K.RFQSINAENTENRR.L
44-56	1551.724	1550.717	1550.696	0	R.FQSINAENTENR.R
44-57	1707.810	1706.803	1706.797	1	R.FQSINAENTENRR.L
44-60	2140.054	2139.046	2139.046	2	R.FQSINAENTENRRLYR.Q
57-68	1551.811	1550.803	1550.868	2	R.RLYRQLLFTADR.V
61-68	963.447	962.440	962.519	0	R.QLLFTADR.V
87-100	1659.849	1658.841	1658.899	2	K.TDDGKLFPQLIKER.D
92-100	1143.608	1142.601	1142.681	1	K.LFPQLIKER.D
99-107	1046.679	1045.672	1045.559	1	K.ERDMVVGIK.V
99-110	1388.667	1387.660	1387.750	2	K.ERDMVVGIKVDK.G

108-133	2690.358	2689.351	2689.356	1	K.VDKGVVPLAGTNGETTTQGLDGLYER.C
111-133	2348.174	2347.167	2347.166	0	K.GVVPLAGTNGETTTQGLDGLYER.C
139-148	1193.613	1192.605	1192.599	2	K.KDGADFAKWR.C
140-148	1065.511	1064.503	1064.504	1	K.DGADFAKWR.C
149-172	2599.283	2598.276	2598.405	2	R.CVLKITDTTPSELAIKENANVLAR.Y
153-164	1288.670	1287.663	1287.692	0	K.ITDTTPSELAIK.E
173-199	2989.471	2988.463	2988.472	0	R.YASICQMHGIVPIVEPEILPDGDHDLK.R
173-200	3145.581	3144.574	3144.574	1	R.YASICQMHGIVPIVEPEILPDGDHDLKR.C
200-207	1026.564	1025.556	1025.496	1	K.RCQYVTEK.V
201-207	870.468	869.461	869.395	0	R.CQYVTEK.V
304-311	801.444	800.437	800.476	0	R.ALQASALK.A
312-330	2148.220	2147.213	2147.058	2	K.AWGGKPENGKACQEEFIK.R.A
322-329	967.523	966.516	966.448	0	K.ACQEEFIK.R
322-336	1750.828	1749.821	1749.883	2	K.ACQEEFIKRALNNSK.A
330-336	802.465	801.458	801.446	1	K.RALNNSK.A
330-341	1289.585	1288.577	1288.667	2	K.RALNNSKACQGK.Y
331-348	1869.949	1868.942	1868.905	2	R.ALNNSKACQGYVSSGDK.G
337-348	1242.642	1241.634	1241.571	1	K.ACQGYVSSGDK.G

342-363	2216.123	2215.115	2215.018	1	K.YVSSGDKGAAGGESLYVANHAY.-
---------	----------	----------	----------	---	----------------------------

97 Start-End, positions of the peptides in the protein sequence; Observed, experimental m/z value; Mr,exp., experimental m/z
98 transformed to a relative molecular mass; Mr, calc., relative molecular mass calculated from the matched peptide sequence; Miss,
99 number of missed cleavage site; Sequence, sequence of the peptide in one letter code.

100

101